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Evolution of Conformational Disorder & Diversity of the P53 Interactome

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Abstract

The tumour suppressor p53 is now known to be an ancient transcription factor that had already evolved interaction sites with its partner protein MDM2 at the dawn of multi-cellular eukaryotic animal life. The billion-year life history of the p53-MDM2 axis has permitted significant time for the proteins to integrate into a distinct range of cellular pathways including binding to hundreds of genomic promoters and regulatory protein-protein interactions with hundreds of distinct functions. This long age of p53 allows us to understand how the protein can regulate a range of functions such as energy generation of the cell, cell motility, genome integrity, virus infection, immune cell response, ageing, and oxidative stress. Due to this deep integration of p53 into the core of eukaryotic life, it is not surprising that the p53 pathway requires inactivation in order for human cancer cells to evade the normal growth controlling processes that have been shaped through evolution by natural selection. This review will focus on the emerging concepts in the protein science field that shed light on p53 protein evolution and function including the nature of thermodynamically unstable regulatory proteins and the growing realisation that the majority of protein-protein interactions in complex eukaryotic cells are driven by intrinsically unstructured and weakly interacting peptide motifs. These concepts help to explain how the vast number of dynamic and specific protein-protein interactions in the p53 pathway evolved, suggest how amino acid modifications like phosphorylation or acetylation in turn evolved to regulate dynamically the p53 interactome, and finally reveal therapeutic strategies for targeting the p53 interactome in human cancers.

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Intrinsic disorder: the rise of a new dogma in protein science that impacts upon our understanding of p53

As biologists or biochemists, we have all learned the structure-function paradigm that proteins display different levels of organisation: a "primary" linear sequence on which depends local spatial "secondary" arrangements stabilised by a three dimensional compact structure, usually referred as globular protein or called *native* fold, i.e. the biologically active form of the protein. Upon this structure-function concept several protein-protein interaction (PPI) models have been developed such as the recent Nobel Prize awarded on solving the structure of a ribosome. It is perhaps not a co-incidence that a major advance on the road to acquire the structure of this prokaryotic multi-protein complex was the decision to switch to purifying ribosomal proteins from thermophilic bacteria which are more suitable to form stable structures using current experimental methodologies. More "unstable" ribosomal complexes from, for example, higher eukaryotes are not suitable for such crystallographic

analysis. What makes these more temperate multi-protein complexes "unstable"?

Despite the fact that the structure-function paradigm can explain the mechanism of many protein–protein interactions, this paradigm has been "modified" and challenged by an increasing awareness of non-folded protein regions exhibiting functionality. For example, comparing the resolution of protein crystal structures and protein sequence banks, only 7% of proteins are devoid of any "disorder" pattern and around only 25% exhibit >95% of their lengths in their 3D structure. This suggest that the vast majority of proteins in the PDB are shorter than their corresponding sequence length [1]. From many such similar clues, there has emerged a new concept in the protein science field that established that there are relatively large regions of functional, intrinsically unstructured regions, or disordered proteins forming the IUP or IDP kingdom.

These natively unfolded components can encompass degrees of disorder from the totally disordered protein, even large ones, to some locally intrinsically disordered region (IDR) that can also be as small as only a few amino acid residues. The extent of disorder can vary widely from transient local unfolding, to partially strictly unstructured regions, to totally unfolded domains, that can be modified by sequence variation in amino acid composition and nearby environment [2, 3]. In human diseases, examples of proteins with extended and large regions of intrinsic disorder include the ARF tumour suppressor, the MDM2 oncoprotein, and the Parkinson disease/cancer associated proteins in the synuclein superfamily.

IUPs throw new light on the flexibility of PPIs and Connectivity of pathways

Since the recognition of their existence, hundreds of articles have described cases of intrinsically unstructured proteins (IUPs), which led to an analysis of the importance of this phenomenon on a genome scale. A study on protein disorder from different kingdoms have shown that the extent of disorder can be as low as 3% in prokaryotes, over 15% in yeast, and over 50% in mammals [4]. This forms large information rich landscape composed of "unstructured" polypeptide sequence in the proteome of higher eukaryotes and forms the basis for expanding substantially the numbers of "connections" between proteins. Indeed, it has been suggested that one reason why higher mammalian life developed, although with a similar number of genes to invertebrates, is the large increase in novel protein-protein connections driven by intrinsically disordered regions, that allow new low affinity, reversible but highly specific, signal transduction systems to emerge.

There are wide correlations between the increasing amount of disorder in the proteome and the complexity of the organism considered. Disordered proteins were shown to hold key positions and play roles in more prominent pathways in higher organisms such as cell signalling, regulation, molecular recognition and control pathways rather than metabolic processes where classic structural domains reign dominant. These signalling pathways require high specificity and, at the same time, low affinity so that, that IUPs can adopt stable folds when interacting with different partners. These properties allow IUPs to nucleate protein hubs that form high interaction networks through an unusually large number of dynamic, unstable, but functional protein-protein interactions [5, 6]. Indeed, IDPs not only enable a single protein to bind a large number of partners (at different times) but also allow the binding of multiple proteins to a common partner. Thus, the lack of "intrinsic" structure becomes a functional advantage and gives novel evolutionary adaptive benefits in fine-tuning or allowing a change in molecular interaction networks. IUPs/IDRs involved in protein-protein interactions display several special features listed below in comparison with "ordered" proteins/regions. These properties emerge in essential requirements for weak, rapid, transient, promiscuous, reversible, specific and also highly regulated protein-protein interactions that so much exploited in signalling and regulation [7]. Understanding protein-protein interactions mediated by IUPs would help to map interactome to decipher complex mechanisms that are involved in pathologies like cancer or neurodegeneration.

Specific properties of IUPs: state-of-the-art interface, folding coupled to binding, preformed structure

IUPs or IDRs are classified by a negative, a non well-defined 3D structure. But this unstructured state is not synonymous with a denatured state, as point mutations can affect the activity thus demonstrating that the IUP or IDR contains somehow the propensity to acquire structure [8]. Disordered proteins showed different chemical and physical features, compared to globular counterparts, including their composition and interface sequences. The overall amino acid composition of an IUP or IDR is enriched in amino acids of high flexibility indexes with disorder promoting amino acid such as Ala, Arg, Gly, Gln, Ser, Pro, Glu or Lys. These confer a high net charge and a low net hydrophobicity to the IUPs/IDRs that ensure an extended state and an inability to fold, naturally. In term of interface area, their hydrophobic residues are more exposed than buried in contrast with the hydrophobic core of a globular protein [9]. As IUPs exploit hydrophobic-hydrophobic interactions and as they exploit a larger relative interface area, they engage a larger number of contacts that drive a better fit with a partner. Moreover IUPs in 70% of the cases use a single continuous segment for partner binding as opposed to

ordered protein interfaces that are more segmented [10].

All of these differences within disordered proteins manifest themselves in interaction energies. Alone, a disordered protein can't necessarily fold into a stable manner because of lower number of inter-residue interactions compared to an ordered protein. In fact IUPs gain stabilisation energy from their intermolecular contacts in the presence of a partner, this binding can induce a large-scale folding of the IUP. Various models emerge from this folding upon binding; such as fly-casting which states that the flexibility of disordered structures enables a weak and not so specific binding to evolve into a folded and thus specific binding to a partner [10, 11]. The principal feature of IUPs is their pliability toward a wide range of partners revealed in multiplicity of accessible structural states separated by low energy barriers. IUPs could adopt different folds that can be a well-defined 3-D structure, when interacting with different partners. Therefore IDRs would possess inherent conformational preferences leaded by a residual preformed structure [9].

Molecular recognition features and short linear motifs

In order to recognise their partners, IUPs often use sequential molecular recognition element/features (MoREs/MoRFs) imparting specificity to PPIs. MorFs are short region of order-promoting residues (10-70 amino acids) flanked by IDRs and are characterised by some aspects previously discussed as they fold upon binding and contain some pre-existing structure [12, 13]. There are three different types of MoREs: those that form an α-helix upon binding with a partner (α-MoRE); those that form β-strands (β-MoRE); and those that form irregular structures (I-MoRE) [12]. It has been shown that short linear motifs or SLiMs (3-10 amino acids) in proteins often fall (around 85%) into locally disordered regions. These short functional sequence patterns capture key residues involved in function or binding. For example they can correspond to a region including a critical site for modifications as phosphorylation or localisation signals like KDEL. Identifying linear motifs could help to find binding partners and explain how one protein is able to bind so many different partners, but also could be instructive about their biological functions and how interaction networks are constructed [14]. So far only hundreds of linear peptides have been identified. There are likely still numerous linear motifs yet to be discovered as they are difficult to discover [15]. Different interaction studies have attempted to capture them computationally at a genome-scale, rather than one by one slow experimental discovery process [15]. In term of evolutionary plasticity, they can be switched on and off quite easily by point mutation. Consequently they are often poorly conserved (as in the striking divergence between the murine and human ARF tumour suppressor), but different species would use the same kind of motifs that have eventually arisen convergently [16].

The disordered binding region (MoRFs) and the linear motif concepts describe molecular interactions on different bases: the first one focuses on the structure and the second approaches the problem through the sequence. However, the interactions described by the two concepts describe two aspects of the same phenomenon as in both cases the interaction is confined to a relatively short, continuous region in one of the partners. The study of MoRF and SLiM connection is still ongoing [17].

Conservation of the interface of IUPs

As the increase in disorder has been shown to correlate positively with regulatory functions that have undergone expansion between bacteria and higher multicellular eukaryotes, a closer look at the evolution of genes coding for intrinsically disordered proteins could explain the changes in an IUP interactome. Disordered regions don't display any significant evolutionary conservation compared to structured regions in proteins, and this makes them relatively difficult to identify. Regions which become ordered upon complex formation are more conserved, especially key residues in an molecular interface [10]. Within a same family of a gene or a single gene, disordered proteins/regions are able to undergo rapid evolution preserving functionality without degenerating into a pseudogene. Faster evolution within these disordered regions compared to globular region, due sometimes to gene duplication, repeat expansion, and mutations explains that IDPs/IDRs are commonly more difficult to find by sequence similarity. We can imagine subsequently that cells possess a stunning evolutionary tool to adjust a protein-protein network or network cross-talk to respond to new selection pressures of the environment or epigenetic signalling pathways that imprint a change in a cell steady-state [18]. Nonetheless, studying the evolution relationship between an IDP and its partners might also be harder to follow linearly as evolution is not necessary unilateral; co-evolutionary adaptive changes of both partner and IDPs can occur [19].

P53 hub, a partially disordered protein

The P53 signalling protein

The tumour suppressor p53 plays a central role in cancer suppression by preventing proliferation of damaged cells with potentially cancer-prone mutations through its ability to act as a DNA-binding protein and transcription factor, whereby it can induce the transactivation of proteins that play a role in cell cycle arrest, apoptosis, senescence, DNA repair or alter metabolism [20-24]. The *p53* gene is

one of the most widely mutated genes in human cancer, it can be observed in over 50% of all human tumours and these tumours respond poorly to therapy [25]. This central player in the cancer control artillery is present in a low activity state and at low levels under unperturbed conditions but becomes rapidly activated in response to a variety of stimuli such as UV light, DNA damage, virus infection, and overexpressed oncogenes [26]. In unstressed cells, p53 is under the negative regulation of the murine double minute clone 2 (MDM2) protein that mediates the ubiquitination and degradation of p53 by the proteasome. P53 transcriptionally activates the *mdm2* gene and because MDM2 inhibits p53 activity, this forms a negative feedback loop that regulates p53 function [27].

P53 structure and PPIs

P53 is an extensively studied modular protein of 393 amino acids which functions rely on various domains (Figure 1): an N-terminal transactivation domain (TAD) subdivided into TAD1 (residues 1-40) and TAD2 (40-61); an adjacent potential conformational element consisting of a proline-rich domain (PRD; 64-92); a large DNA-binding domain (DBD; residues 93-293); a tetramerisation domain (4D; 325-356) connected to the DBD via a flexible linker; and a basic C-terminal regulatory domain (CTD; 367-393) [28]. These domains regulate its function as a stress-activated sequence-specific DNA-binding protein and transcription factor. The primary amino-acid sequence of p53, especially the TAD region, contains many post-translational modifications (Figure 1) that have a crucial role in regulating p53 interactions thus its stabilisation and activation [29, 30]. Therefore p53 integrates signals of multiple signalling pathways by post-translational modifications [31, 32]. According to disorder prediction software (Figure 1), around 50 to 60% of its residues are considered within an intrinsically disordered structure. Two regions, amino acids 25-50 at the N-terminus, 325-350 at the C-terminus, have a higher tendency of being structured and are flanked by disordered region; another segment around amino acids 150-190 could be a disordered region connecting two structured domains at both sides within the DBD.

p53 has been extensively studied leading to an important number of discovered PPIs that could either be created between a disordered p53 segment and an ordered partner, either between an ordered p53 segment and a disordered partner, or also between two disordered p53 segments as it is likely in the p53 tetramer formation. So far 485 partners according to BioGRID database, 266 direct protein interactors according to the Human Protein Reference Database, 303 according Interologous Interaction Database (SwissProt ID: P04637, 315 from POINeT database, have been brought forward by different experimental methods (Figure 2). Both TAD and CTD are

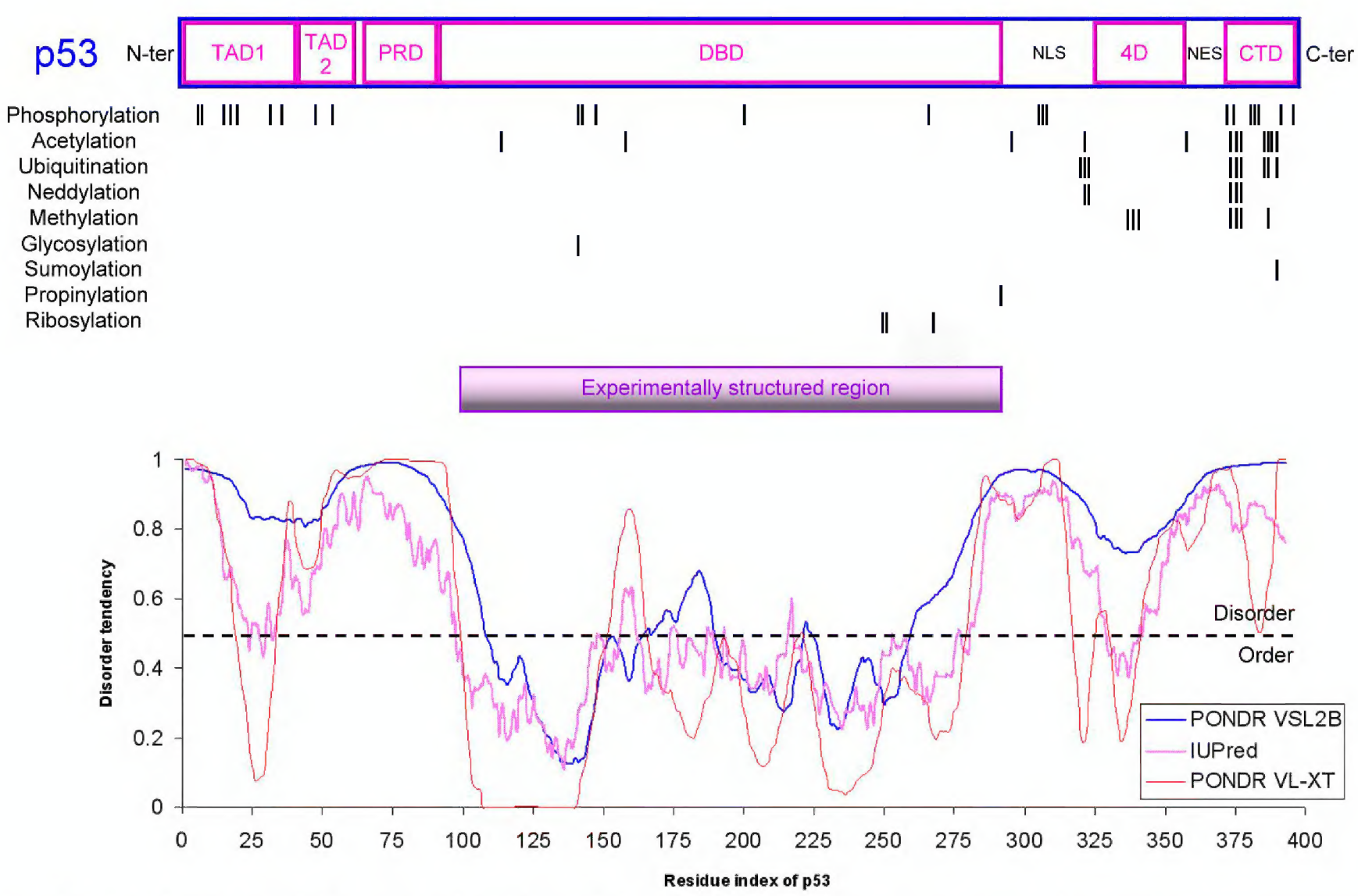

Figure 1. Sites of post-translational modifications and structure of p53. Post-translational modification sites are represented by vertical ticks [31, 32]. Experimentally structured region corresponds to crystallised DNA-binding domain. The dashed line at 0.5 of Y-axis is threshold line for disordered/structured residues. Residues with a score above this latter are predicted disordered and with a score below are predicted to be ordered. DisProt VSL2B [33], PONDR VL-XT [34] and IUPred [35] correspond to the prediction from the predictor of the same name. All features are represented to scale as indicated by the horizontal axis.

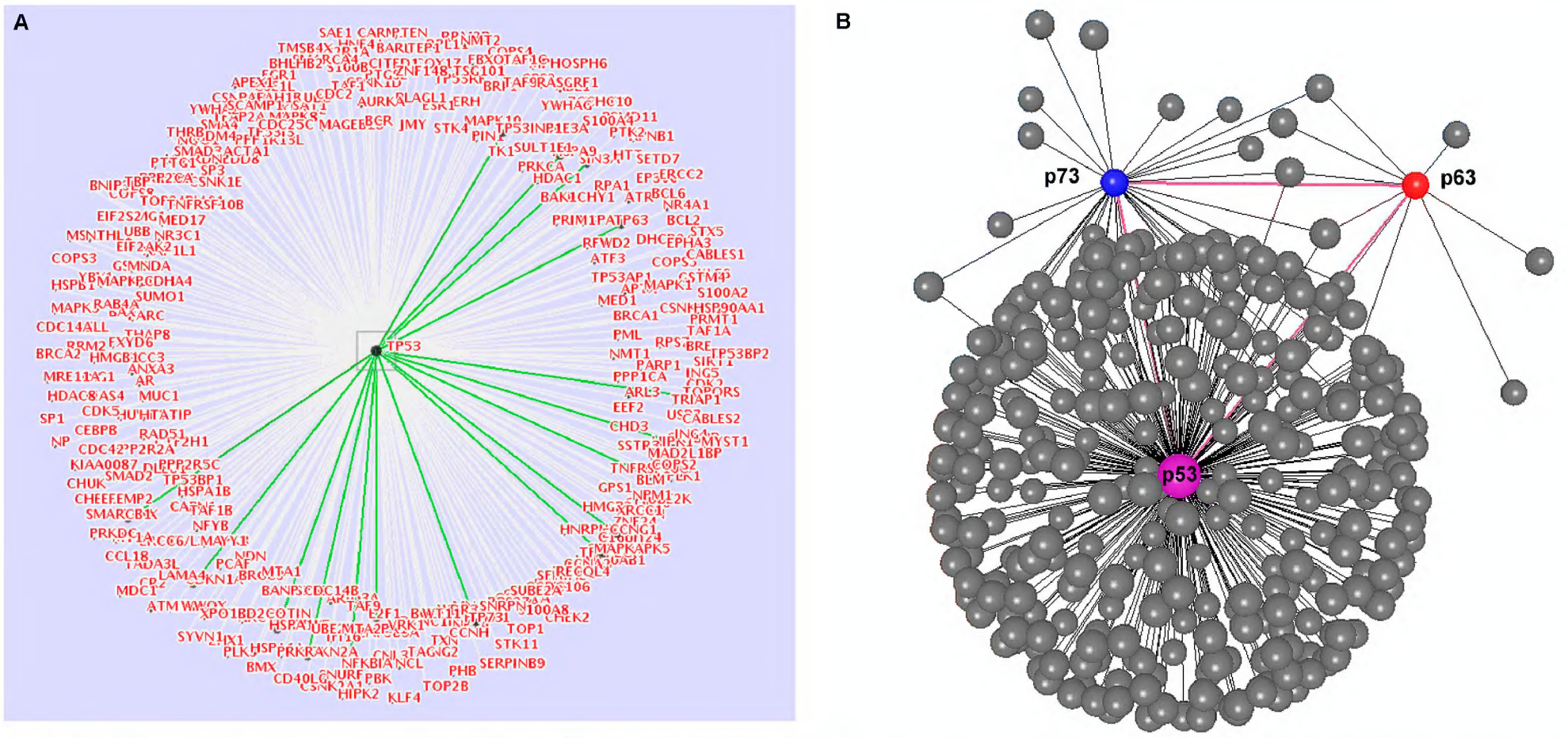

Figure 2. P53 protein interaction network. A) Experimental human p53 non-redundant interactors build with POINeT whose protein-protein interaction data sources are from BIND, AfCS, HPRD, BioGRID, MPact, DIP, IntAct, NCBI interactions, MINT, MIPS databases (http://poinet.bioinformatics.tw). **B)** P53, p63 and p73 interaction networks from Interologous Interaction Database visualised with NAVIGatTOR 2.1.13 software. This p53 family network displays interplays as it exhibits common interactors between the two and/or three of them. I2D (Interologous Interaction Database) is an on-line database of known and predicted mammalian and eukaryotic protein-protein interactions (http://ophid.utoronto.ca).

especially multi-functional binding sites for a multiple of interacting proteins. For examples among many others, p53 TAD region interacts with TFIID, TFIIH, MDM2, RPA, CBP/p300 and CSN5/Jab1 and the CTD domain interacts with GSK3β, PARP-1, TAF1, TRRAP, hGcn5, TAF, 14-3-, S100ββ [36].

Post-translational modifications

In addition to the vast number of protein-protein interactions, p53 is also subjected to covalent modifications such as phosphorylation that can regulate its specific activity [37]. Post-translational modifications (PTMs) are essential for signalling as they affect protein turnover, stability, localisation but also interaction properties and thereby affect deeply the protein function. Recent studies have shown that most phosphorylation sites, as well as acetylation and methylation sites are not localised within ordered regions but are located in unstructured parts of proteins. Indeed, p53 has many phosphorylation sites in its intrinsically disordered regions [31], with one of the most recently identified phosphorylation sites in the conformational flexible DNA binding domain whose phosphorylation drives wt-p53 into an unfolded mutant-like state [38, 39]. By extension it is likely that other modifications such as ubiquitination, sumoylation, or neddylation will be more often found in unstructured protein segments and these modifications might similarly prime p53 for conformational changes that impact on its function or stability. These different PTMs create a pattern, just like a "binding code", as they can modify the local charge density and hydrophobicity, and in this manner affect the structure and the folding properties of the local chain and its surface [11]. Overall, PTMs are providing dynamicity in the PPIs mediated by IDRs/IUPs and can adapt the function accurately in space and time scales to fulfil different and specific biological cell outcomes.

IDRs of p53 and their bound-structures

PONDR VL-XT disorder prediction software was applied to the p53 sequence (Figure 1) and resulted in the identification of two regions with a MoRE pattern within p53 [3, 13]. The first is located between residues 17-35 which overlaps with p53 binding region to MDM2 (residues 13-29) and which undergoes a disorder-to-α-helix transition [13, 40]. The second MoRE resides between positions 374-392, which partly cover residues 367-388 known to fold upon binding to S100β, another binding partner of p53 [41]. In addition, the software confirmed an order region with only marginal instability (residues 100-290) matching almost the identified DNA binding domain; and an α-β-MoRE tetramerisation domain (residues 320-360) which undergoes mutually induced folding by self association in the tetrameric state by dimerisation of the dimers [3, 13]. Similar results could be obtained with ANCHOR software [42], which seeks to predict disordered binding regions able to undergo a disorder-to-order transition via binding to a structured partner (Figure 3).

The Nter acidic activation domain (TAD) of p53 consists of a completely unstructured region when

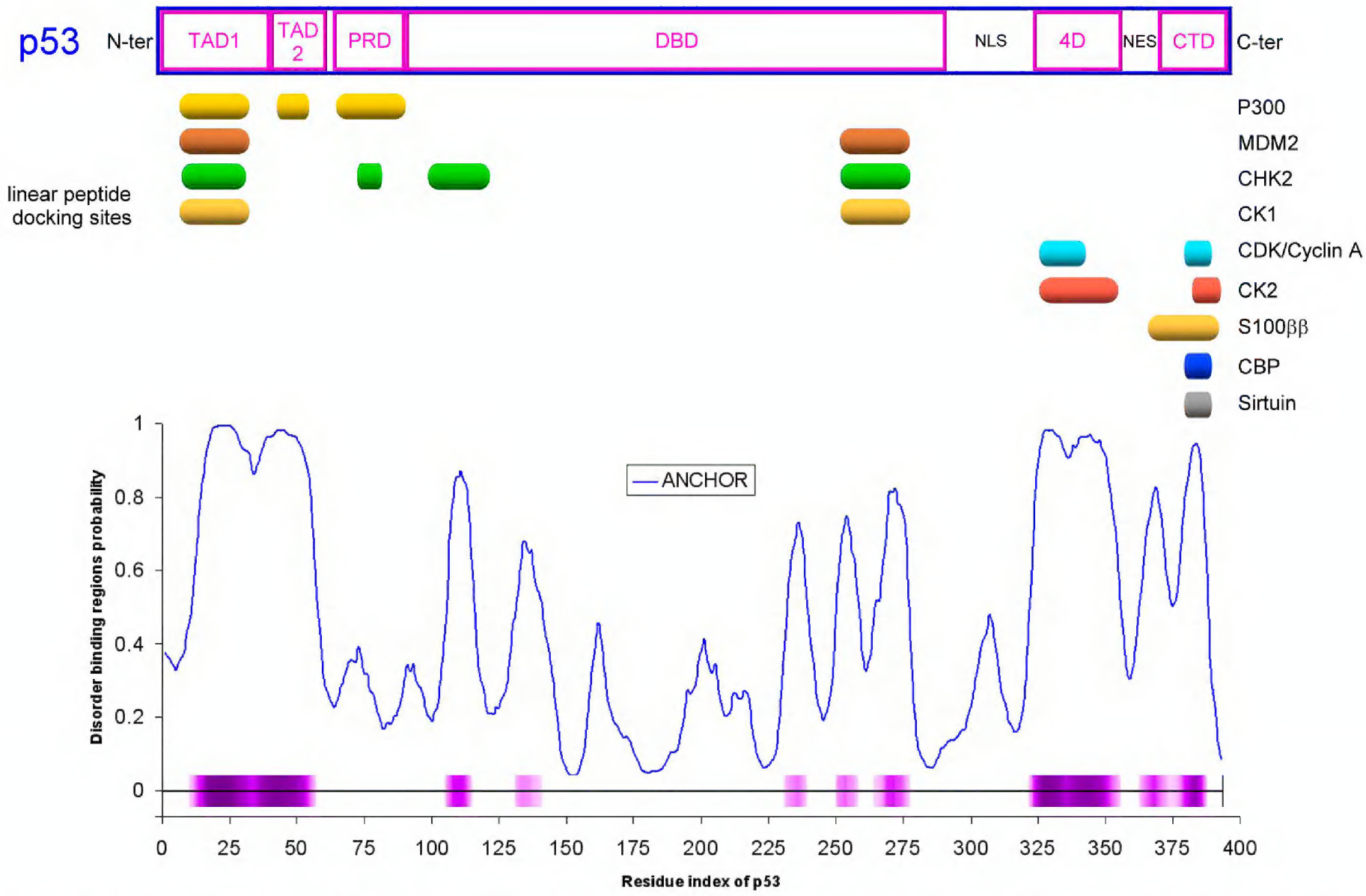

Figure 3. Linear peptide docking/binding sites in p53 are correlated with MoRE-pattern binding regions. The overlapping but distinct linear binding motifs for p53 function regulators include the acetyltransferase p300 and deacetylase Sirtuin, the E3 ubiquitin ligase MDM2, the protein kinases CHK2, CK1, CDK/Cyclin A, CK2, the activator CBP and the repressor S100β [31, 43].

free in solution and fold upon binding to their targets. This domain is promiscuously a binding site for the co-activator p300, components of the transcription machinery and the negative regulators MDM2/MDM4 [44]. In complex with 109-residue amino-terminal of MDM2, a 15-residue binding peptide of p53 TAD adopts an α-helical conformation (2.5 turns) showed by X-ray crystallographic studies. Binding between MDM2 cleft and the hydrophobic face of the p53 α-helix relies on three deeply inserted amino acids of p53 – 19, 23 and 26 – which are involved also in p53 transactivation [40]. In nuclear magnetic resonance (NMR) and residual dipolar coupling studies, a similar single turn helix at the same amino acids 22-24 is transiently formed when p53 is free in solution, showing that this unstructured region displays a preformed structure [45, 46]. Indeed the full-length p53 TAD was shown by NMR to be populated with preformed structures – an amphiphatic helix formed by residues 18-26 and two nascent turns by residues 40-44 and 48-53 [46]. The TAD2 including these two latter turns have been shown to fold into amphiphatic α-helices upon binding to replication protein A and a subunit of yeast TFIIH [44].

Interestingly, same highly basic IDR of p53 (residues 374-388), which is subject to extensive regulatory post-translational modifications (Figure 1), is able to fold in a partner-depending manner into three different secondary structures in the bound state (Figure 4): a α-helix when interacting with S100β [41], a β-sheet with sirtuin [47], an irregular structure with CBP [48] and another irregular one with cyclin A2 [49]. Analysis indicated that the same residues are involved in the four interfaces suggesting that this region includes a "code" read differently by the different partners which induces different binding characteristics [9, 50]. The function of the CTD is only partially understood with some controversy in the literature about its role *in vivo*.

Quaternary structure of p53

The structure of full length p53 in a tetrameric state bound to DNA and TAD-binding Taz2 domain of p300 (which both reduced the flexibility of p53) and free in solution has been unveiled by a combination of different techniques such as small-angle X-ray scattering and electron microscopy [44, 51]. The free full-length p53 displays a cross-shaped tetramer with loosely coupled DBD and extended TAD and CTD. Free p53 is a heterogeneous population with mainly open conformation. Interestingly murine p53 showed a different tetrameric structure formed through N and Cter when cryo-EM is applied to the recombinant protein. This may reflect the flexibility in the p53 tetramer or it may be an artefact of the methodology. In the presence of DNA, tetrameric p53 enfolds around

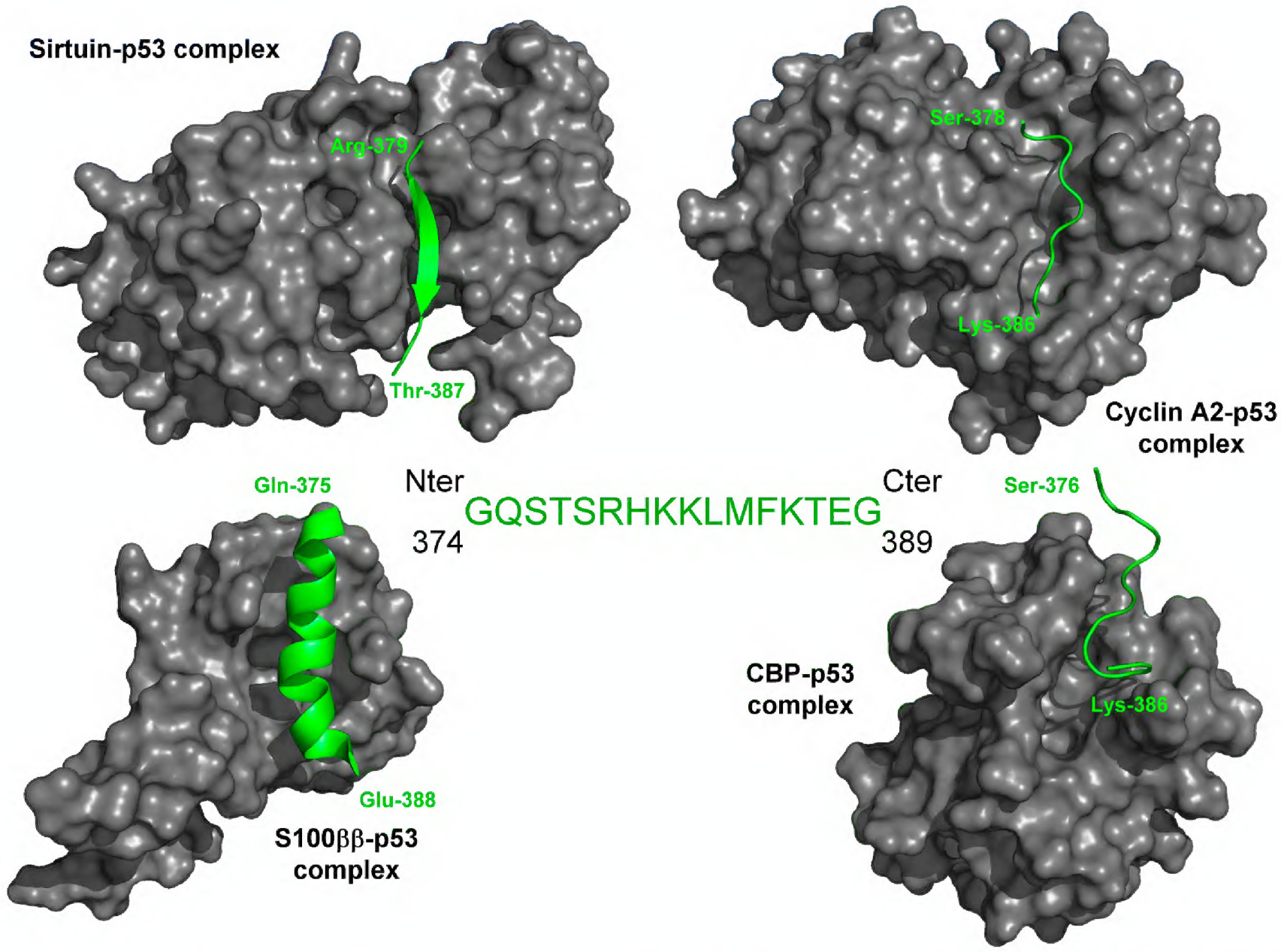

Figure 4. Different secondary structures adopted by the same C-terminus segment of human p53 when bound to different partners. Region between amino acids 374 and 389 (green) have been shown to become a helix when binding to S100ββ (PDB: 1dt7), a sheet when binding to sirtuin (1ma3), and a coil with two distinct backbone shapes when binding to CPB (1jsp) and Cyclin A2 (1h26). Same residues of p53 are used to different extents by the four interfaces.

the DNA helix rendering the overall structure more rigid. The extension of the Nter is a common feature in both free and DNA-bound p53 representation, allowing multiple regulatory interactions [44, 52].

Targeting PPIs to turn on p53

Primarily structure-based drug design were developed by focusing on well-ordered active sites, however in the past decade a new approach of disorder-based rational drug design has lead to alternative promising drugs [53]. Identification of small molecules or peptide aptamers that specifically target interaction interfaces involving unstructured regions may embody a novel drug discovery strategy [14]. Due to its involvement in cancer, the p53 pathway is universally a star pupil in developing innovative strategies and mechanisms for drug therapy [54, 55]. Indeed for p53 targeting, cis-imidazoline analogs, Nutlins have been developed to mimic the p53 α-helix within the TAD (Figure 5). Thus these small molecules are able to bind really specifically to the hydrophobic cleft of MDM2 and block the interaction between MDM2 and p53 which activates this latter [56, 57]. New leads for therapeutic use could be obtain by designing small molecules targeting PPIs, approach which requires learning more about them through intrinsically disorder structures.

P53 throughout the ages: the origin of p53's disorder

A family of genes: sisters but not twins

P53 has been show to belong to a superfamily of related transcription factors with the discovery of two homologues p63 and p73 whose structure and function are analogous but not identical to those of p53. Their genes encode for multiple p63, p73 or p53 proteins containing different protein domains (isoforms) due to multiple splicing, alternative promoter and alternative initiation of translation. Studies on the p53 gene structure established that it encodes 9 different protein isoforms [58-60]. The human p53 orthologues displays a dual structure conserved in Drosophila and zebrafish, which underlies the importance of the various roles of isoforms [61]. P53, p63 and p73 appear to possess overlapping and distinct functions: p53 is essential as a tumour suppressor, as a pro- or anti-ageing factor, and it can induce cell death and growth arrest; p63 is essential for ectoderm development; and p73 might regulate both stress response with tumour suppression role as well, and development/differentiation [62-64]. However p73 and p63 have been both shown to act as tumour suppressors and are being associated with new pathways linked or not with p53 [65]. As they are able to

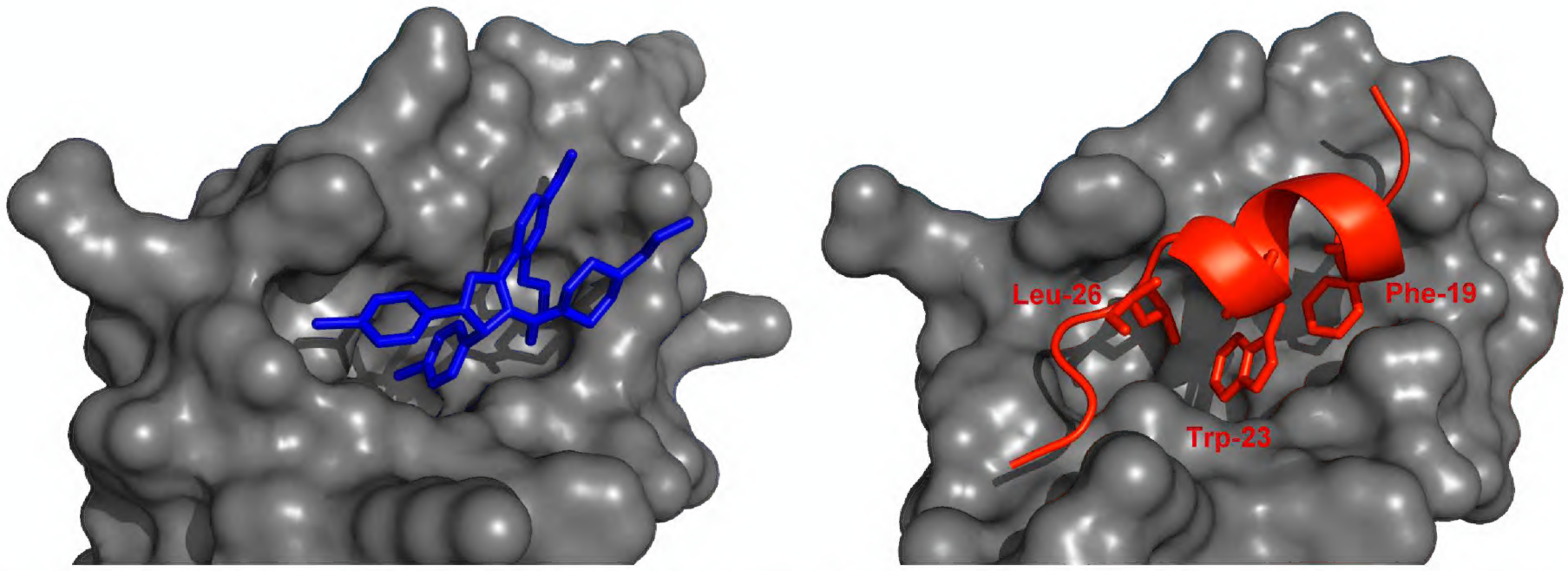

Figure 5. Structural application to p53 structure-based drug therapies. The crystal structure of nutlin (blue) in complex with MDM2 (grey) mimics the three residues from p53 (red) that are involved in p53-MDM2 interaction (left: 1rv1; right: 1ycr).

target common genes and to form heterocomplexes, there is no doubt that this trio participate in a complex interplay by interfering and collaborating with one another.

From their disorder profiles (PONDR VL-XT data), p63, p73 and p53 overall disordered percentages are respectively 50.59%, 55.03% and 49.11%. Analysis of these disorder profiles against the schematic representation of p63 and p73 [66] revealed a similar disorder/order pattern for both proteins (Figure 6). Respectively for p63 and p73, three or two ordered potential binding regions lie within the disordered TAD. Both PRDs and regions between 4D and sterile α motif (SAM) domain are highly disordered in contrast with the structured DBDs. Nevertheless DBDs show a region of 100 amino acids similar to p53 DBD that have a tendency to be disordered and which could undergoes a transition disorder to order upon DNA binding. In comparison with p53, the NLS is also disordered but display a sharp ordered motif and 4D domains are also disordered with two binding motifs. Lastly, p63 and p73 possess a unique ordered SAM domain and their CTDs are disordered with a large ordered motif or two closed motifs within these latter similarly to p53 CTD.

The p63 and p73 proteins possess only one TAD homologous to the TAD1 of p53. Thus, the N-terminus is the least conserved domain among the p53 family members, but at the same time, both p63 and p73 can activate sets of p53 target genes, and moreover key residues for some interactions such as p53-MDM2 are conserved within the family. The helical propensity of the region previously discussed in p53 structure properties within its TAD is similar for p53 and the analogous region of p73, but is lower for p63, which could explain different behaviour towards partners. In addition, PTMs as phosphorylation can perturb or enhance helicity of this region that will enable dissociation with some partners or tighter association with some others [67].

The main difference between p53 family members resides in the C-terminus (Figure 1 and 6) as p53 contains a basic domain natively unfolded and the site of numerous PTMs, whereas p63 and p73 possess a sterile ordered α motif and an unstructured region C-terminal to the SAM domain which is called transcriptional inhibitory domain (TID). This C-terminus can be spliced in some isoforms and these latter truncated isoforms are more transcriptionally active which underlies the regulatory function of the C-terminus similarly than the p53-CTD [66, 68]. The SAM domain is a globular domain composed of a five-helix bundle topology and is important for the stability of the oligomerisation domain as it stabilised it [69]. This C-terminus ordered domain of p63/p73 is essential of stabilising the architecture as a tetramer, which is lost in mammalian p53 during evolution as this latter has acquired through regulating PPIs within its disordered CTD [70, 71]. Another divergence between members is the presence of an additional helix in the oligomerisation domain of p73, which is conserved in p63. TD domain that is essential for the formation of tetramers is the only active form of p53 for stable DNA-binding and subsequent gene transactivation. Differences in the structural domains within p53 family members within the same organism are responsible for the diversity of their various functions, so it can be extent easily to p53 from different organisms, whose structure evolution lead to modify its interactome and thus its functions.

Origins, evolution and role of disorder in p53

Overall, p53 forms an ancient regulatory network with MDM2 and is fundamentally linked to adaptative stress responses governing in a first instance germ-line regulation, then was shaped to control fidelity of reproduction, development and longevity, to finally specialise as a tumour suppressor to protect cells from aging and cancer, in not only gametes but in every somatic cells in higher organisms [72-74]. Neither the p53 nor MDM2 family is known to be present in bacteria, archaebacteria, yeast, or

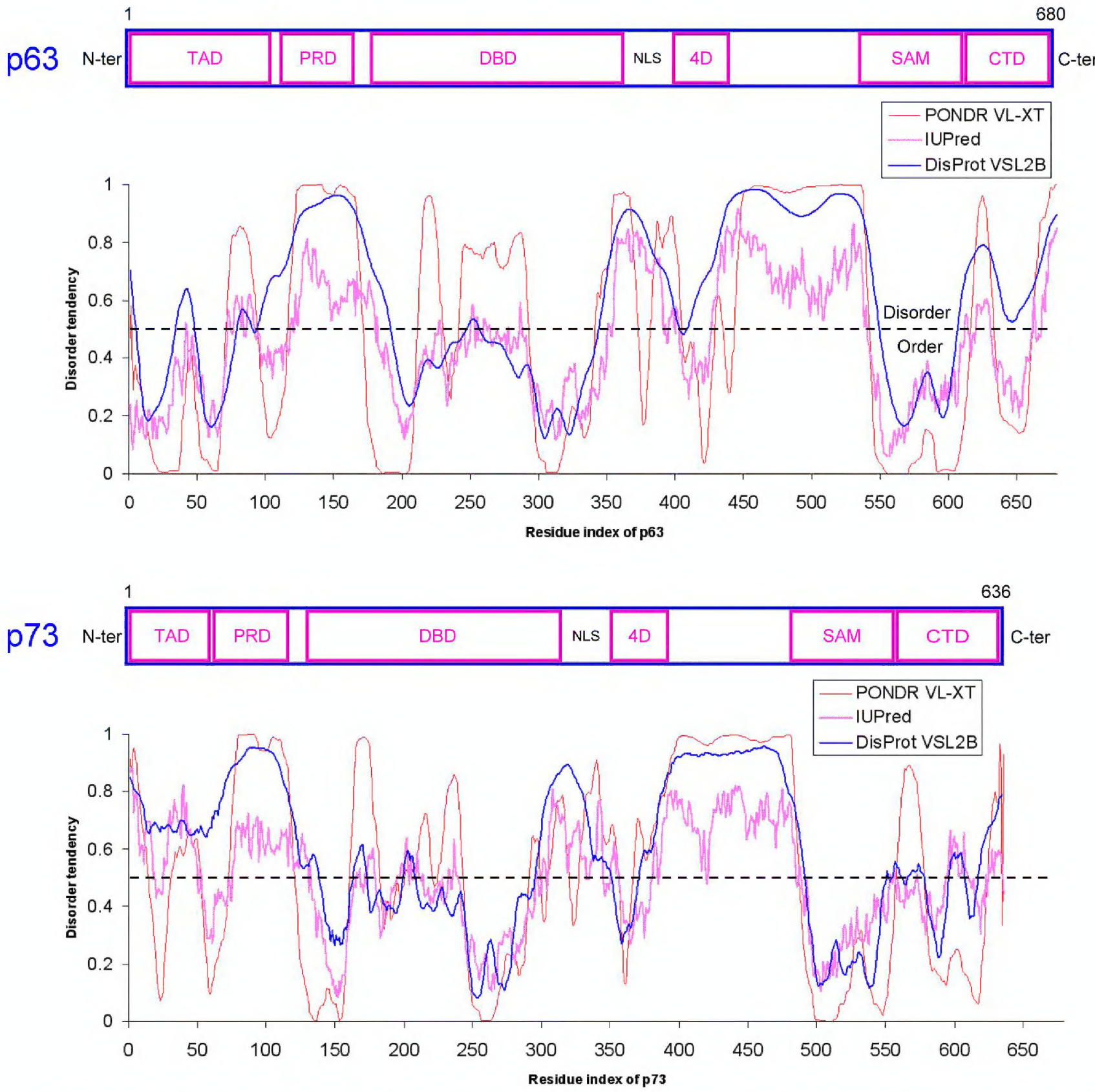

Figure 6. Schematic representation and disorderness of p63 and p73. The dashed line at 0.5 of Y-axis is threshold line for disordered/structured residues. Residues with a score above this latter are predicted disordered and with a score below are predicted to be ordered. DisProt VSL2B [33], PONDR VL-XT [34] and IUPred [35] correspond to the prediction from the predictor of the same name. All features are represented to scale as indicated by the horizontal axis.

plants, but it is now believed to have emerged in response to selective pressures on early multicellular organisms in the Animalia kingdom (metazoans). Indeed, p53 (27% identity with human p53) and MDM2 have both been shown to be present in Placozoans (*Trichoplax adherans*), one of the simplest multicellular organisms as it displays three different cell types [75]. This discovery pushed back the evolutionary history of p53 and MDM2 as co-factors to a billion years. However, equally amazingly, although p53 and MDM2 generally persist together throughout insect and urochordate life, they have been strikingly lost in the famous 20th century model organisms used by scientists, *Drosophila melanogaster* and *Caenorhabditis elegans*. The loss of p53 and MDM2 in these two model organisms is a blow to our 'having a ready-to-use genetic' system to dissect out the fundamentals of the p53-MDM2 axis, but nevertheless highlights that we could perhaps develop other invertebrates as genetic models to study p53 and MDM2 in the decades to come.

Interestingly, other genomes have been used to examine the origin of p53 within the Animalia branch. Two p53 paralogs (a p53-like sequence and a p63/73-like sequence) are present in a choanoflagellate (*Monosiga brevicollis*), a free-living unicellular and colonial eukaryotes considered to be the closest living relatives of the animals [76, 77]; also a p53-like protein could have been discovered in a amoeba genome (*Entamoeba histolytica*) which is an anaerobic parasitic protozoan [78]. Lastly, a putative p53 homologue has been shown in maize with possible involvement during DNA replication and growth control at an embryo germination level [79, 80]. Consequently, the emergence of p53 family might have predated the appearance of metazoans, but seems to have appeared within eukaryotic cells, perhaps set apart

from prokaryotic cells by the presence of a chromatin-constrained nucleus, which interestingly is essential for p53 subcellular distribution that is central for its regulation and functions.

Lastly, it is interesting to underlie that from a complexity point of view *Monosiga* should be closer to the common ancestor form of the p53-like protein, but the p53-like protein from *Trichoplax adherans* is closer to any other p53-like protein from human through to choanoflagellate (Figure 7). The extent of disorder seen in the p53 amino acid sequence might have been quite prominent a billion years ago (Figure 7), which implies the protein had already found a way to use this strategy as a means for regulating flexible, low affinity, but specific protein-interaction hubs as it started on the road to imbed itself into many dominant pathways in eukaryotes.

A

Organism	NCBI accession number	Protein length (residues)	Overall % disorder	Overall % identity	DBDs % identity compared to one organism		
Homo sapiens	NP_000537.3	393	49.11	100	100	40	21
Rattus norvegicus	NP_112251.2	391	52.43	76	90	43	21
Danio renio	NP_571402.1	373	52.55	47	69	40	24
Caenorhabditis elegans	NP_001021478.1	644	37.89	4	8	19	16
Drosophila melanogaster	NP_996267.1	495	34.55	11	18	24	11
Ciona intestinalis	NP_001122370.1	489	52.35	29	38	38	25
	NP_001071796	419	36.52	27	39	36	21
Nematostella vectensis	EF424411.1	378	50.53	23	36	38	20
	EF424412.1	369	31.17	21	32	32	17
Trichoplax adhaerens	XP_002113784.1	576	37.50	27	40	100	23
Monosiga brevicollis	XP_001747656.1	571	49.21	18	21	23	100

B

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Homo_sapiens                ----LSSSVPSQKTY-QGSYGFRLGF----------------LHSGTAKS
Rattus_norvegicus           ----LSSSVPSQKTY-QGNYGFHLGF----------------LQSGTAKS
Danio_renio                 ----PTSTVPETSDY-PGDHGFRLRF----------------PQSGTAKS
Ciona_intestinalis_489      ----SKPTIPPSTDY-PGEWDFQINFGE--------------ATESAPKS
Ciona_intestinalis_419      ----TEVSLLPNNEY-PGIYNFEINFGE--------------KTESAPKS
Nematostella_vectensis_378  ----HLPTIPSTAEY-PGELGFCVSFGP--------------PTESASKS
Trichoplax_adhaerens        ----RDPSLPSNAEY-IGNFGFDIAID---------------QNDNPTKA
Nematostella_vectensis_369  ----HHVIAPSSDEV-PGEYSFKLTLET--------------QPKKVAN-
Drosophila_melanogaster     DIQIQANTLPKLENHNIGGYCFSMVLD-------------------EPPK
Monosiga_brevicollis        -----DLTLLQNGSAPIPISSNSLHVPC--------------RWPHFDVL
Caenorhabditis_elegans      QMDFTETNVPFDGNDESSNLAVRVQSDMNLNEDCEKWMEIDVLKQKVAKS
                                                                      :

Homo_sapiens                VTCTYSPA---LNKMFCQLAKTCPVQLWVD--------------------
Rattus_norvegicus           VMCTYSIS---LNKLFCQLAKTCPVQLWVT--------------------
Danio_renio                 VTCTYSPD---LNKLFCQLAKTCPVQMVVD--------------------
Ciona_intestinalis_489      AQYTYSPI---INKLFVKMNVTCPIKFKCA--------------------
Ciona_intestinalis_419      APFTYSYS---LQKLFVKMNENCPIKFRCS--------------------
Nematostella_vectensis_378  ATWTYSEK---CKKLYVNLASFCPIKFKTT--------------------
Trichoplax_adhaerens        TNNTYSTM---LKKLFIKMECLFPIHITIERMDYTFKIAYGSLATRRNCN
Nematostella_vectensis_369  PDWIYSTS---QNKLYIKPQTPCPMKFSVT--------------------
Drosophila_melanogaster     SLWMYSIP---LNKLYIRMNKAFNVDVQFKS--------------------
Monosiga_brevicollis        LMIQYSPQ---LGRFFVHVNADVVLKIVLAR--------------------
Caenorhabditis_elegans      SDMAFAISSEHEKYLWTKMGCLVPIQVKWKLDKR-----------------
                                 ::         ::  .       :..

Homo_sapiens                -----STPPPGTRVRAMAIYKQSQHMTEVVRRCPHHER----CSDSDGLA
Rattus_norvegicus           -----STPPPGTRVRAMAIYKKSQHMTEVVRRCPHHER----CSDGDGLA
Danio_renio                 -----VAPPQGSVVRATAIYKKSEHVAEVVRRCPHHER----TPDGDNLA
Ciona_intestinalis_489      -----RPPPNGCVVRVMPVFKRPEHVTDIVTRCPNHK----IPDQAQHIP
Ciona_intestinalis_419      -----PQPPSGCVIRAIPVFEKPNNVTEIVTRCFNHRNEC-RTESSDSNT
Nematostella_vectensis_378  -----VKPPPGSYLRGVAVFKGSTNLHDIVKRCPNHM-----ETSQDGQE
Trichoplax_adhaerens        QLIIPGEPPANSYIRAYVMYTKPQDVYEPVRRCPNHAL-----RDQGKYE
Nematostella_vectensis_369  -----GCVPPGTFIRAIPIFKLPEHAKDVVRCCPNHTLLE---QSNRDHP
Drosophila_melanogaster     -----KMPIQPLNLRVFLCFSN--DVSAPVVRCQNHLS-V-EPLTANNAK
Monosiga_brevicollis        ------APPKGTDLVFRLRYALPEHRKTRVETCVTHQQ----AGSHFFGA
Caenorhabditis_elegans      -----HFNSNLSLRIRFVKYDKKENVEYAIRNPRSDVMKCRSHTEREQHF
                                                  :    .    :     .
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```
Homo_sapiens                  PPQHLIRVE--GNLRVEYLDDRNT----FRHSVVVPYEPPE----VGSDC
Rattus_norvegicus             PPQHLIRVE--GNPYAEYLDDRQT----FRHSVVVPYEPPE----VGSDY
Danio_renio                   PAGHLIRVE--GNQRANYREDNIT----LRHSVFVPYEAPQ----LGAEW
Ciona_intestinalis_489        HSQHLIRAEMPGENPAIYNVAMD-----GRENVAVMFERPQ----IGAEY
Ciona_intestinalis_419        PNSHLIRVESKSNN-IQYCLTHE-----GRECVVVPYEPPH----SGSEY
Nematostella_vectensis_378    KVSHFMRSNNPSAR---YNVCPES----GRHSILIPYTGPQ----VGTEF
Trichoplax_adhaerens          SSDHILRCE---SQRAEYYEDTS-----GRHSVRVPYTAPA----VGELR
Nematostella_vectensis_369    AMAHFIRSD---NPRAEYERCAQS----GRLSVKIPFHVTQGSI-SEEII
Drosophila_melanogaster       MRESLLRSE---NPNSVYCGNAQGKGISERFSVVVPLNMSRSVTRSGLTR
Monosiga_brevicollis          PHNHLMSIN---REHVTYDTDSTG-----HHYARVALDQFP----FTDNV
Caenorhabditis_elegans        PFDSFFYIR---NSEHEFSYSAEK----GSTFTLIMY--------PGAVQ
                                  ::           :                :

Homo_sapiens                  TTIHYNYMCNSSCM--GGMNRRPILTIITLEDSSGNLLGRNSFEVRVCAC
Rattus_norvegicus             TTIHYKYMCNSSCM--GGMNRRPILTIITLEDSSGNLLGRDSFEVRVCAC
Danio_renio                   TTVLLNYMCNSSCM--GGMNRRPILTIITLETQEGQLLGRRSFEVRVCAC
Ciona_intestinalis_489        TTVLYKFMCLSSC--VGGINRRPLNAVFNLENAEGQVLGRRVVEVRVCSC
Ciona_intestinalis_419        MALLYRFMCLSSCRTETGINRRPLLTIFNLESETGELLGKRVVSTRICAC
Nematostella_vectensis_378    VTEMFAFMCFSSCP--SGPSRRPVEIIFTLE-KDGQTLGRQVVEIRVCAC
Trichoplax_adhaerens          STLLYQFMCFSSCS--GSINRRPIELVITLENGTN-VLGRKKVEVRVCAC
Nematostella_vectensis_369    VHELFSFVCNNSCG---GLNRRAIQIVFTLEQAVAVSCWRCSIETRVCAC
Drosophila_melanogaster       QTLAFKFVCQNSCI-----GRKETSLVFCLEKACGDIVGQHVIHVKICTC
Monosiga_brevicollis          YSVPLRFHCFSSCP--GSIARRMMQLMVYLEHSEH-ILGITSVDCRCCAC
Caenorhabditis_elegans        ANFDIIFMCQEKCLDLDDRRKTMCLAVFLDDENGNEILHAYIKQVRIVAY
                                    : * ..*       :     :.  :               :  :

Homo_sapiens                  PGRDRRTEEENLRKKG
Rattus_norvegicus             PGRDRRTEEENFRKKE
Danio_renio                   PGRDRKTEESNFKKDQ
Ciona_intestinalis_489        PGRDRSQEEKRKRTAE
Ciona_intestinalis_419        PGRDRTQEEEKKNVT-
Nematostella_vectensis_378    PGRDRKSDEKLLLGEA
Trichoplax_adhaerens          PGRDRSNEERAAMKSE
Nematostella_vectensis_369    PGRDSKQDNEAVSRPG
Drosophila_melanogaster       PKRDRIQDERQLNSKK
Monosiga_brevicollis          PGRDRLSAEKRQQRNS
Caenorhabditis_elegans        PRRDWKNFCEREDAKQ
                              * **
```

Figure 7. A) P53-like proteins among various species throughout evolution. Either protein, nucleotide or gene accession numbers of protein sequences which were used are specified. The overall percentage of disorder was calculated with PONDR VL-XT and identity scores were obtained from multiple sequence alignments using CLUSTAL 2.0.12. **B)** Multiple sequence alignment of human p53-related DBDs ("*" for identical residues in all sequences; ":" conserved substitutions; "." semi-conserved substitutions).

Conclusion: drug discovery and disordered motifs

We have described the emerging view of the linear or disordered domain in the formation of protein-protein interactions in eukaryotes and how this can impact on our understanding of the protein interaction landscape that captures p53. Defining the global interactome of a target protein is an emerging technological challenge in the field of systems biology. Such knowledge has a potentially high impact in human medicine where rate-limiting nodes can be identified as a focus of novel diagnostic or therapeutic advances. One significant insight into the nature of a protein-protein interaction in the last decade has been the concept that a large proportion of the polypeptide sequence information in higher eukaryotes is intrinsically disordered thus providing a template for "weak" regulatory, combinatorial, and specific PPIs to occur in signal transduction [81]. A second advance was the realisation that a significant number of PPIs occur via a linear amino acid motif that provides opportunities for hotspots to be identified in a PPI and thus the emerging view is that drugging PPIs form a new and untapped landscape in the drug discovery field [82]. Thus, identifying approaches that can capture the dynamic linear motif interactome of a target protein provides another avenue to build systems map of a protein. Such screens have expanded on the potential number of interactors to hundreds and highlight the utility in approaches that capture weak but regulatory PPIs.

The p53 and MDM2 axis form an evolutionary ancient

prototype protein-protein interaction pair that exploits the intrinsically disordered motif of one protein and a peptide-binding pocket interface in another. MDM2 binds to an intrinsically disordered peptide motif in the p53 TAD1 domain that can be mimicked by the small molecule Nutlin-3. This small molecule can activate p53 [56] and recent clinical trials of an MDM2 peptide-binding mimetic drug-lead show promising responses in clinical trials of human sarcoma containing an amplified *mdm2* gene [83] and how a p53-dervied peptide motif can be developed into an oncogenic protein inhibitor. Although there are almost 500 p53 interacting proteins published with little information on the mode of binding at the PPI interface, there are a growing list of PPIs that form crystal structures giving rise to structural modelling possibilities (Figure 4 and Tables 1 and 2). As the drug discovery field is acknowledging ever more that protein-protein interactions form an untapped landscape for therapeutic development [84], it is highly likely that other oncogenic protein-protein interactions between linear intrinsically disordered motifs of p53 and peptide binding pockets in target proteins will emerge into drug discovery programmes in the future.

Table 1. List of p53 peptides or domains co-crystallised with binding partners.

Molecule	Ligand	PDB code
P53 N-terminal peptide	MDM2	1YCR
	MDM4	3DAB
	histone acetyltransferase p300	2K8F
	replication protein A 70	2B3G
	RNA polymerase II transcription factor B	2GS0
P53 C-terminal peptide	S100ββ	1DT7
	Sirtuin 2	1MA3
	CREB-binding protein	1JSP
	Cyclin A (-CDK2)	1H26
	14-3-3	3LW1
	USP7	2FOJ/2FOO
	histone acetyltransferase GCN5	1Q2D
	methyltransferase SET9	1XQH
P53-derived O-GlcNAc peptide	O-GlcNAcase	2YDR
Monomethylated p53 peptide	methyltransferase SMYD2	3S7D
P53 C-terminal dimethylated peptide	DNA repair factor 53BP1	3LGF
P53 DNA binding domain	DNA repair factor 53BP1	1GZH
P53 DNA binding domain	SV40 large T-antigen	2H1L
P53 tetramerisation peptide	P53 tetramerisation peptide	2J0Z

Table 2. List of peptide and peptide-derived molecules that can activate p53 through PPI disruption.

Molecule	Ligand	Drugging strategy
P53 N-terminal peptide [40]	MDM2	p53 hydrogen-bond-surrogate α-helix [85] p53 hydrocarbon-stapled α-helix (3V3B) [86] p53-mimicking small molecule Nutlin (1RV1) [87] cell-penetrable spiroligomer α-helix [88] p53-based small molecule MI-219 [89] p53 peptidomimetic trisaccharide [90]
P53 N-terminal peptide [91]	MDM4	Optimized p53 peptide [92] targeting MDM2/MDM4
P53 peptide from tetramerisation domain [93]	CDK2-Cyclin A/B	Based on CDK2 docking site
P53 C-terminal peptide [94]	Core domain of P53 mutant	Activates p53 through displacement of the negative regulatory C-terminal domain
CDB3 peptide [95]	P53 mutant	Based on p53C-53BP2 complex (1YCS)
Optimized AGR2-binding penta-peptide [96]	P53	Stabilises p53 and increases its nuclear localisation
PKCα-derived peptide [97]	Tetrameric p53	Regulate oligomerisation state of p53

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